AD_____
(Leave blank)

Award Number:
W81XWH-08-1-0694

TITLE:
The Role of Central Metabolism in Prostate Cancer Progression

PRINCIPAL INVESTIGATOR:
Thomas P. Conrads

CONTRACTING ORGANIZATION:
University of Pittsburgh

REPORT DATE:
October 2009

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
12-10-2009	Annual	15-09-2008 – 15-09-2009

4. TITLE AND SUBTITLE

PC074313, The Role of Central Metabolism in Prostate Cancer Progression

5a. CONTRACT NUMBER
PC074313

5b. GRANT NUMBER
W81XWH-08-1-0694

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Thomas P. Conrads, PhD

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pittsburgh
204 Craft Avenue
Pittsburgh, PA 15213

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We hypothesize that by enriching the diet with ω-3 PUFAs PCa tumor progression will be significantly reduced. Patients with localized PCa will be enrolled in a randomized, double-blinded phase-I clinical trial in which they will be given ω-3 fish oil or control oleic acid supplements 5 weeks prior to prostatectomy. We will evaluate differential protein and phosphopeptide expression in PCa cells, obtained from laser capture microdissected tissue, by mass spectrometry. As primary endpoints, we will compare proliferation and apoptosis in tumors from each group using immunohistochemistry and DNA nick end labeling. As secondary endpoints, we will compare serum prostate specific antigen and hormone levels. We will also measure levels of dietary PUFAs in red blood cell membranes as well as FASN and PUFA metabolic products in prostate tissue using high performance liquid chromatography, mass spectrometry, and gas chromatography.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**		12	Thomas P. Conrads
					19b. TELEPHONE NUMBER *(include area code)* 412-641-7556

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

	Page
Introduction……..	**4**
Body……..	**4-7**
Synergy Statement……	**5**
Key Research Accomplishments……..	**5-8**
Reportable Outcomes……	**8**
Conclusion……	**8**
References……	**8**
Appendices……	
Supporting Information……..	**9-12**

Introduction

Currently, no curative therapy exists for advanced, metastatic prostate cancer (PCa). However, given the long latency period of PCa progression coupled with the close association between aging and disease incidence, therapies that impede cancer growth would make PCa clinically irrelevant. Dietary intervention represents one such therapy. Epidemiological studies demonstrate that intake of ω-3 polyunsaturated fatty acids (PUFAs) decreases PCa risk while ω-6 intake increases risk. Work from our laboratories and others suggests that the metabolites of dietary ω-3 and -6 PUFAs directly affect PCa and the ability to do so depends on intake and metabolic enzyme expression. Omega-3 and -6 PUFAs compete as substrates for cyclooxygenase-2 and 15-lipoxygenase-1, both elevated in PCa; these enzymes convert ω-3 PUFAs to anti-tumorigenic metabolites and ω-6 to pro-tumorigenic ones. PCa cells also have elevated fatty acid synthase (FASN). FASN regulates the expression of a myriad of genes, including the PUFA metabolic enzymes Δ-5 and -6 desaturase and phospholipases that liberate arachidonic acid, which together may affect the pool of PUFAs. In addition, ω-3 inhibits FASN, inducing apoptosis in PCa cell lines. These data provide evidence that ω-3 PUFAs, through anabolic and catabolic fatty acid pathways as well as possibly other pathways, modulate PCa. We hypothesize that by enriching the diet with ω-3 PUFAs PCa tumor progression will be significantly reduced. Patients with localized PCa will be enrolled in a randomized, double-blinded phase-I clinical trial in which they will be given ω-3 fish oil or control oleic acid supplements 5 weeks prior to prostatectomy. We will evaluate differential protein and phosphopeptide expression in PCa cells, obtained from laser capture microdissected tissue, by nanoflow reversed-phase liquid chromatography coupled online with high-resolution and –accuracy tandem mass spectrometry. As primary endpoints, we will compare proliferation and apoptosis in tumors from each group using immunohistochemistry and DNA nick end labeling. As secondary endpoints, we will compare serum prostate specific antigen and hormone levels. We will also measure levels of dietary PUFAs in red blood cell membranes as well as FASN and PUFA metabolic products in prostate tissue using high performance liquid chromatography, mass spectrometry, and gas chromatography.

Body

Over the current performance period from 9/15/08 to 9/15/09 we have been pursuing IRB and FDA approval and submission and resubmission of revised protocols. However, we were able to get the protocol in place which had to be reapproved by the Department of Defense (DoD). We then resubmitted the protocol as an amendment to the University of Pittsburgh institutional review board (IRB) which is now approved. We have acquired the required fish and oleic acid capsules which are appropriately stored and will be dispensed in the University of Pittsburgh dispensary where randomization will take place and the capsules delivered to the clinical nurse/coordinator in Urology clinic. All the members involved in the trial are in constant touch either via e-mail and/or telephone calls with updates if any. One of our collaborating PI, Dr. Beth Pflug has moved to Indiana University and grant negotiation between DoD and Indiana University is in progress. We expect to start recruiting patients by the beginning of 2010.

Furthermore, major progress has been made in extending and standardizing our tissue proteomic methodologies and bioinformatics workflows that will be leveraged to accomplish the primary goals of the proposed activities in the W81XWH-08-1-0694 sub-award and these are summarized below. Because we have not accrued prostate cancer patients due to delays associated with IRB and FDA approval, we report on accomplishments that we have made that point to our general progress in clinical tissue handling, including laser capture microdissection (LCM), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and bioinformatics for differential protein quantification from the tumor microenvironment.

Synergy and Statement of Work

The three PIs of this grant, Drs. Conrads, Kelavkar and Pflug have been working together effectively to issues related to protocol approval and clinical trial management, demonstrating the commitment of this team. The three PIs have established a secure database of all the forms (data from primary and secondary end-points) on the University of Pittsburgh Medical Center network and currently available in Clinical Trial Management Application (CTMA) for internal study number 07-164. This database can be accessed remotely from anywhere in the world. For security reasons, we have provided a list of all users and their associated access level (i.e., read-only, add, modify). This will enable the involved individual/s log-on remotely into CTMA via the UPMC network by connect@upmc.com and upload individual data after the analyses is complete in the fillable forms in the web-site. This way all data will be blinded and collected in one place and retrieved, mined, analyzed and reported seamlessly. This database is the essential component for accomplishing all tasks outlined in the Statement of Work for this grant and it is now active and all three PIs are fully trained on its use.

Key Research Accomplishments

Sample Preparation for Global Proteomics

To correlate the biomarker-tumor connection, tumor biopsies have to be performed and other biochemical or imaging methods, such as IHC, need to be performed to validate the site of origination of a given protein biomarker. The initial and groundbreaking proteomic study from cancer tissue was toward investigation of prostate cancer (PCa) and benign prostatic hyperplasia (BPH) utilizing cells procured from the same archived prostate thin tissue section (1). Several hundred proteins were identified from these samples, including a variety of prostate-related proteins such as prostatic acid phosphatase and prostate-specific antigen. This analysis also resulted in the identification of proteins distinct to each histopathology, such as growth differentiation factor 15, which was solely identified from prostate cancer cells and not in those from the BPH or stromal regions.

Our group has worked to develop and standardize a single-tube methodology for efficient digestion of proteins from cells acquired by LCM from thin tissue sections. The protocol that we have developed efficiently extracts proteins from either fresh-frozen or formalin-fixed, paraffin-embedded (FFPE) tissue. In the case of FFPE tissue, the protocol begins with deparaffinization (with xylene) and rehydration (through graded ethanol solutions) of FFPE thin tissue sections cut by microtome on specialized LCM slides. The deparaffinized tissue sections are lightly stained with hematoxylin and eosin (H&E) for pathological review and LCM. Laser capture

microdissection is conducted using an upright laser microscope (Leica) to directly harvest cells from pathologically distinct regions from the tissue microenvironment. This procurement is facilitated by interrogating a polyethylene polymer on which the tissue thin section lies with a pulsed UV laser. This process enables an efficient excision of the area of tissue impinged by the laser that, with the aid of gravity, results in collection of defined regions of tissue from the microenvironment directly into a microcentrifuge collection tube – the entire collection process is aided by a wireless drawing pen that is used to draw the borders for LCM directly on an active LCD screen display of the thin section image. Upon completion of drawing the borders representing the tissue region of choice, an area calculation is provided to inform of the region (or regions) in square microns that are designated for harvest. This area calculation is critical for downstream proteomics, as this is a primary metric for normalizing sample input for LC-MS/MS analysis. Importantly, the microscope system enables image capture prior to and after the LCM process **(Fig. 2)**.These images are archived for instances where follow-up pathological assessment may be required. To increase protein yield and MS signal strength from small populations of cells, we have developed a "single-tube" protocol for proteomic preparation of cells captured by LCM **(Fig. 3)**. This methodology results in the efficient generation of whole proteome digests from LCM-gathered cells from tissue. The protocol involves heating the sample in 30 μL of collection buffer (20% CH_3CN, 100 mM NH_2HCO_3, pH 8.0) in a step-wise fashion, first at 95 °C for 60 min then 65 °C for 2 hr, where after it is cooled to 37 °C and directly digested with sequencing grade porcine trypsin for 16 hr.

This protocol is scalable such that 96 tissue samples can be denatured and digested in parallel with the use of a thermocycler. The combined use of an organic solvent (20% acetonitrile) in the buffer, along with the step-wise heating of the sample, represents a MS-friendly, hybrid heat-induced (HI) and enzyme-mediated (EM) antigen retrieval methodology that results in liberation of readily identifiable tryptic peptides by tandem MS (MS/MS).

As a proof of principal to demonstrate the efficiency of our tissue proteomic workflow, this protocol was utilized to conduct a proteomic analysis of ten FFPE clinical samples selected from the University of Pittsburgh Health Sciences Tissue Bank representing defined stages of neoplastic progression of Barrett's metaplasia to high grade dysplasia to adenocarcinoma. Laser capture microdissection was conducted as described above to procure cells from each histological region **(Fig. 2)**. Approximately 40,000 cells (e.g. $6x10^6$ μm^2 x 8 μm thick) per sample were collected directly into buffered acetonitrile, treated with the hybrid HI/EM antigen retrieval described and digested with sequencing grade trypsin. *Importantly and central to the success of this workflow, cell collection, protein solubilization and trypsin digestion are all carried out in a single tube.* The LCM digests were vacuum dried, resuspended in 15 μL of 0.1% trifluoroacetic acid TFA) and each analyzed in duplicate by nanoflow LC-MS/MS with a hybrid linear ion trap-Orbitrap MS. Our strategy for analysis of clinical sample sets such as this is to ***code and randomize*** the samples so as to blind the mass spectrometrist to the nature and order of the samples to be analyzed by LC-MS/MS. This strategy is utilized to minimize propagation of error in the statistical and bioinformatic correlative analyses that may be influenced by biases such as analysis order, batch effects or time of analysis. Shown in **Fig. 3A** is a basepeak mass chromatogram from an LC-MS/MS analysis that represents 20,000 Barrett's metaplasia cells on column and demonstrates that our tissue proteomic preparation procedure produces sample digests of high complexity that do not possess incompatible characteristics that interfere with high-resolution nanoflow chromatography. Our protocol involves addition of 60 fmol each of

three stable isotope standard (SIS) peptides that elute at an early, middle and late time point during our chromatographic gradient of 180 min **(Fig. 3B)**. These SIS peptides enable us to monitor and critically evaluate various analytical processes of the LC-MS/MS analysis, including chromatographic column performance, retention-time variations, mass measurement accuracy and MS/MS efficiency. These analytical parameters are evaluated by reconstructing the mass chromatograms of the SIS peptides from which the retention time and integrated chromatographic peak areas are extracted and the average observed mass to charge ratio (*m/z*) for each of the SIS peptides is calculated. These peptides also serve as "landmark" features that are utilized to facilitate chromatographic alignment of LC-MS datasets from multiple samples. This accurate alignment is critically important for label-free quantitative software applications, such as SIEVE. The retention time variation was less than 1% **(Fig. 3C)** with an average drift in mass measurement accuracy of less than 3 parts-per-million (ppm) (data not shown) in our LC-MS/MS analyses of the archival esophageal tissue samples, and point to the stability and robustness of our tissue proteomics workflow. The primary tandem MS data for this analysis were searched with SEQUEST operating on a 72 processor Beowulf cluster in our laboratory against the human proteome database (UniProt) for peptide identification. The MS/MS data are also searched against a "decoy" human proteome database that has the protein sequences reversed. Searching the data against a decoy database enables adjustment of the SEQUEST X_{corr} and $\square_{cn}$ filter criteria to minimize false peptide discovery (2). Each analytical dataset is searched against a decoy database for adjustment of our filtering criteria to maintain false discovery rates of less than 1%. This process enables us to retain only the highest quality peptide identifications to be included in our downstream bioinformatic pipeline. The peptide lists from each duplicate LC-MS/MS analysis of the LCM-procured cells are integrated using a set of computational and database tools that we have developed in-house in Microsoft Access. The primary peptide export data from SEQUEST are integrated and a set of Access queries enable automated protein identification from the raw peptide sequence output. We have an integrated and fully automated set of database tools that enable strict accounting for whether a peptide sequence is unique to or shared across multiple protein accessions. The analysis that we have conducted resulted the identification of over 2000 unique peptides per LC-MS/MS analysis of 20,000 cells on column with an average relative standard deviation (RSD) of peptide identification rate of 15% across all injections, and an average of nearly 700 proteins identified per tissue sample.

A quantitative estimate of the relative abundance of proteins from such data sets is obtained by comparison of the number of sequenced peptides per protein (e.g. spectral count). This correlate is now commonplace in proteomics and popularized by the term "label-free quantitation" where it has been analytically demonstrated by a number of investigations that if a protein is present in greater abundance, it will be identified through a relatively greater number of peptide MS/MS sequencing events in one sample versus another, provided that the samples have been carefully normalized for equivalent proteomic digest input into the LC-MS/MS workflow (3, 4). Differences in protein abundance from the BE-, HGD- and EAC-derived cells were thus determined from their spectral count values. To determine statistically significant differentially expressed proteins from each histology (e.g. BE vs. HGD vs. EAC), a Kruskal-Wallis non-parametric analysis of variance (ANOVA) test was used. This enables identification of proteins whose abundance levels change in a statistically significant manner. The confidence interval was set to 95% and significance level was set as $p < 0.05$. Those significant differentially distributed proteins were utilized for further supervised hierarchical cluster analysis. Clustering algorithms split large groups of measurements into subgroups based on the degree of similarity between

members of each subgroup. Degree of similarity can be measured in many ways, but is generally a measurement approximately equivalent to a correlation coefficient. After putting individual measurements into subgroups, hierarchical clustering algorithms then place the subgroups into larger groups, put those larger groups into still larger groups, and so on. The results of hierarchical clustering can be represented by dendograms or "heat maps." Observations' similarities were measured by eleven distance functions, then seven linkage criteria were employed for determining pair wise distances between samples. All of the dendograms were verified by calculating the cophenetic correlation coefficient. The closer the value of the cophenetic correlation coefficient is to 1, the more accurately the clustering solution reflects original data. The cluster with the cophenetic correlation coefficient closest to 1 was selected and is shown in **Fig. 4.** A clear pattern is evident representing abundance level alterations of proteins that cluster with each stage along the metaplasia-dysplasia-adenocarcinoma sequence of neoplastic progression. This approach enabled identification of a large number of differentially abundant proteins along the axis of neoplastic progression, including cytokeratins and intermediate filament proteins, differentiation markers, proteins involved in signal transduction and cell cycle regulation, growth and angiogenic factors, matrix-degrading proteases, and proteins with tumor suppressive and oncogenic potential.

Reportable Outcomes

A secure database of all the forms (data from primary and secondary end-points) on the UPMC network and currently available in CTMA for internal study number 07-164.

Conclusion

We have made substantial progress in development and standardization of our tissue proteomic methodologies and workflows. We are now focused on standardizing a workflow for efficient extraction and analysis of phosphopeptides from LCM-gathered cells from tissue, which we expect to complete in the next reporting period. Through these efforts, we will be poised to conduct the global proteomic and phosphopeptide analysis to understand the key pathways and signal transduction events that underlie the apparent growth inhibitory affect of ω-3 PUFAs on PCa tumor progression.

References

1. Hood BL, Darfler MM, Guiel TG, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. Mol Cell Proteomics. 2005;4(11):1741-53.

2. Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods. 2007;4(3):207-14.

3. Liu H, Sadygov RG, Yates JR, 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem. 2004;76(14):4193-201.

4. Patel V, Hood BL, Molinolo AA, et al. Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression. Clin Cancer Res. 2008;14(4):1002-14.

Supporting Information

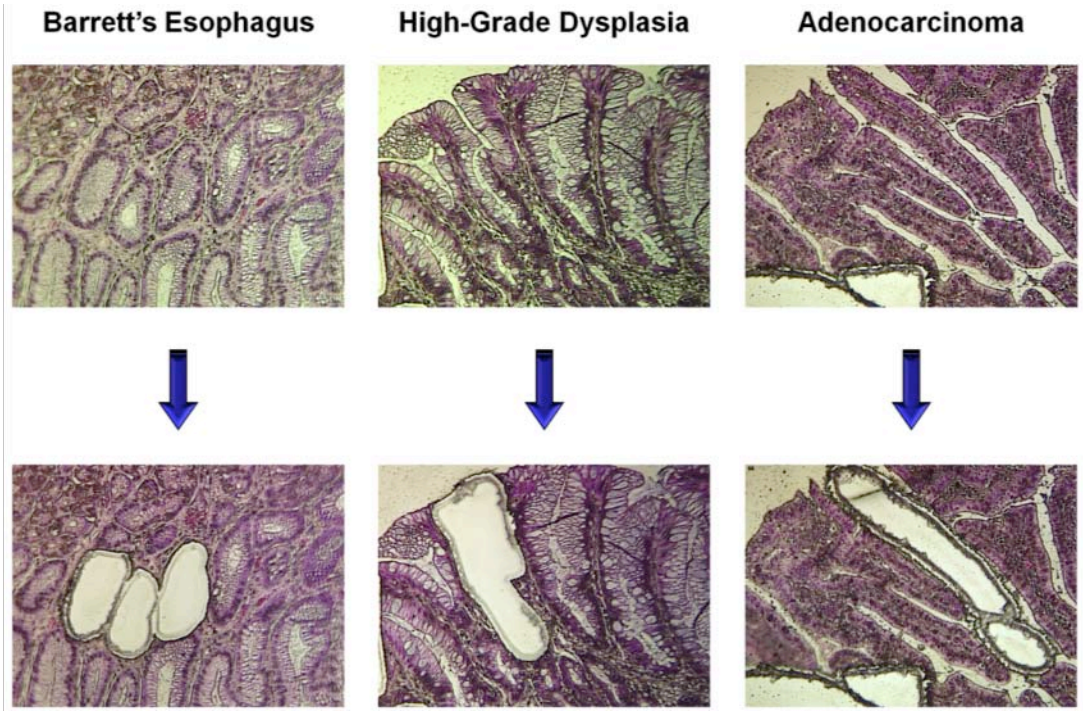

Fig.1: Laser capture microdissection (LCM) of cells from formalin-fixed paraffin-embedded esophageal tissue sections. *Shown are representative hematoxylin and eosin-stained tissue sections containing histological regions displaying Barrett's metaplasia (top left panel), high grade dysplasia (top middle panel) and esophageal adenocarcinoma (top right panel). Shown in the bottom panels are tissue sections after cells from each histology have been procured.*

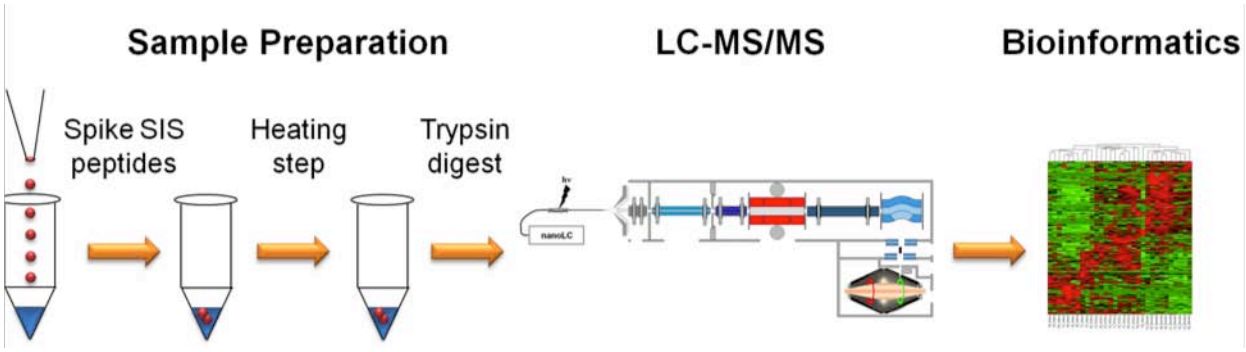

Fig.2: Experimental workflow for tissue proteomics. *The step-wise heating of samples in 20% buffered acetonitrile followed by direct trypsin digestion represents a MS-friendly, hybrid heat-induced (HI) and enzyme-mediated (EM) antigen retrieval methodology resulting in liberation of readily identifiable tryptic peptides by tandem MS. Differential protein abundances are elucidated by spectral counting and utilized in downstream statistical and cluster analyses.*

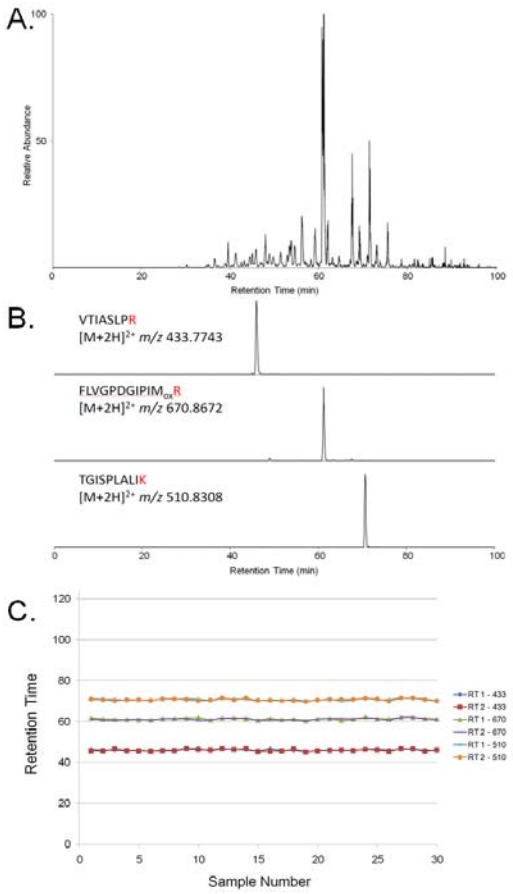

Fig.3: LC-MS/MS of laser-capture microdissected cells from Barrett's esophagus tissue. *Shown is a basepeak mass chromatogram of 20,000 cells from BE (A), reconstructed mass chromatograms of three stable isotope peptides added at 30 fmol per injection (B) and a plot of retention time for three stable isotope standard peptides in a series of 60 LC-MS/MS analyses of archival esophageal patient tissue samples (C). Less than 1% drift in retention time was observed over the 30 samples injected in duplicate (RT1 & 2), indicating robust chromatographic performance over 3 weeks of analyses.*

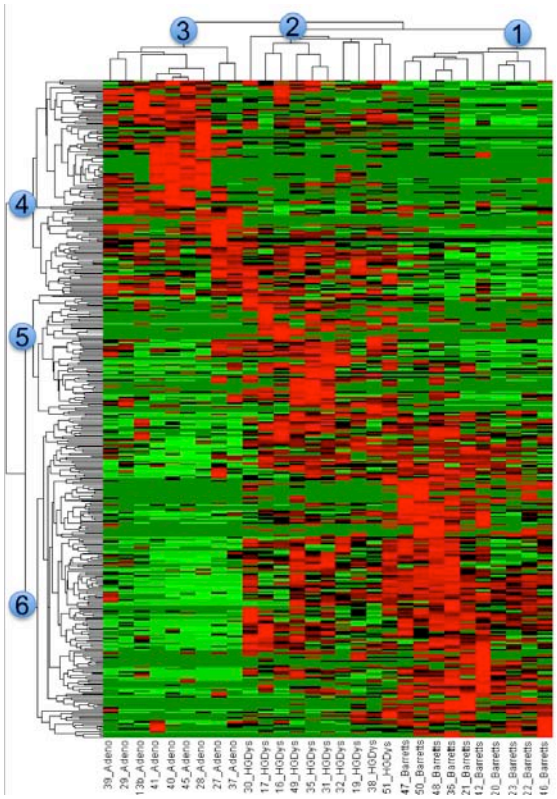

Fig.4: A heat map representation of results from a supervised cluster analysis of 408 significantly differentially abundant proteins identified from laser-capture microdissected cells from Barrett's esophagus, high-grade dysplasia and esophageal adenocarcinoma archival tissues. *Protein abundances are plotted as mean spectral count where red represents proteins with a normalized mean spectral count value greater than 1.5 and green less than 1.5 where significance ($p<0.05$) was determined by the Kruskal-Wallis test. Clear patterns of protein abundance level can be seen to correlate with Barrett's metaplasia (nodes 1&6), high-grade dysplasia (nodes 2&5) and adenocarcinoma (nodes 3&4).*